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(57) Abstract

RXR is a nuclear receptor that plays a central role in cell signaling by pairing with a host of other receptors. Previously, 9-cis-retinoic acid (9cRA) was defined as a potent RXR activator. Here we describe Vitamin F (phytanoic acid), a unique RXR effector identified from organic extracts of bovine serum by following RXR-dependent transcriptional activity, and compositions containing Vitamin F.

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COMPOSITION COMPRISING VITAMIN F

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to chlorophyll metabolites and their derivatives as essential fatty acids. More specifically, the compounds phytenic acid and phytanic acid, and derivatives thereof, have been found to modulate the activity of RXR transcription factors, which in turn regulate the activity of various genes. Thus, phytenic acid, phytanic acid and their derivatives are important dietary components and also find application as ingredients of media for *in vitro* culture of mammalian cells.

Description of the related art

Each article of the scientific and patent literature cited herein is incorporated in its entirety by reference by such citation.

Chlorophyll is best recognized as an energy transducer in plants that captures sunlight for oxygen, sugar, and lipid synthesis and thereby establishes the foundation for animal food chains. Phytol metabolites may now strengthen this link between heterotrophs and autotrophs by integrating the dietary state of the animal with RXR-dependent signaling systems to balance the lipid stores in adipose tissue against cellular needs. Insights into the functions of phytol metabolites may emerge from comparisons with linoleic acid and other unsaturated fatty acids that are important dietary factors synthesized by plants (Burr and Burr, *J. Biol. Chem.* 82, 345-367 (1929) and *J. Biol. Chem.* 86, 587-621 (1930); Aaes-Jorgensen, *Physiol. Rev.* 41, 1-41 (1961)).

Although they serve as important precursors for prostaglandin synthesis (Bergstrom, *Recent Prog. Horm. Res.* 22, 153-175 (1966), unsaturated fatty acids may

also share equally crucial roles as receptor signals. For example, linoleic and arachidonic acids activate PPARα with a potency of 30 μM (Gottlicher et al., *Proc. Natl. Acad. Sci. USA* 89, 4653-4667 (1992); Banner et al., *J. Lipid Res.* 34, 1583-1591 (1993); Keller et al., *Proc. Natl. Acad. Sci. USA* (1993)). Linoleic acid may contribute as much as 20% (40 μM) of the total fatty acids (200 μM) in human or rat sera (Swell et al., *J. Nutr.* 74, 148-156 (1961); Scully et al., *N. Engl. J. Med.* 302, 37-48 (1980)).

Nuclear receptors are transcription factors that regulate gene expression in response to lipophilic ligands such as steroid hormones (Yamamoto, *Annu. Rev. Genet.* 19, 209-252 (1985)). Ligand binding increases the receptor affinity for hormone responsive DNA elements (HRES) near target genes that promote specific transcriptional control (Glass, *Endocrinol. Rev.* 15, 391-407 (1994)). A large family of receptors coordinates cell physiology through these hormone-regulated gene networks (Evans, *Science* 240, 889-895 (1988)). The kindred includes structurally related "orphan" nuclear receptors whose activators are unknown (O'Malley, *Endocrinology* 125, 1119-1120 (1989) and O'Malley, *Mol. Endocrinol.* 6, 1359-1361 (1990)). Although many orphan receptor genes have been isolated by low-stringency hybridization techniques (Arriza et al., *Science* 237, 268-275 (1987)), some, like COUP and HNF-4, initially were described as transcriptional regulators for specific genes (Wang et al., *Nature* 340, 163-166 (1989); Sladek et al., *Genes Dev.* 4, 2353-2365 (1990)).

Activators for orphan receptors have been found by testing compounds in cells transfected with the corresponding receptor and HRE-linked reporter genes (Giguere et al., *Cell* 46, 645-652 (1986); Green and Chambon, *Nature* 324, 615-617 (1987)). Aldosterone, retinoic acid, and ecdysone are some of the ligands

matched with receptors via these "cis-trans" assays (Arriza et al., *Science* 237, 268-275 (1987); Giguere et al., *Nature* 330, 624-629 (1987); Petkovich et al., *Nature* 330, 444-450 (1987); Koelle et al., *Cell* 67, 59-77 (1991)). The nanomolar affinities of these ligands contrast with the micromolar amounts of fatty acids or prostaglandin J_2 required to activate PPARα and PPARγ, respectively (Gottlicher et al., Proc. Natl. Acad. Sci, USA 89, 4653-4667 (1992); Keller et al., *Trends Endocrinol. Metab.* 4, 291-296 (1993) and *Proc. Natl. Acad. Sci. USA* 90, 2160-2164 (1993); Forman et al., *Cell* 83, 803-812 (1995b); Kliewer et al., Cell 83, 813-819 (1995)). Similarly, metabolites of farnesyl pyrophosphate (farnesoids) are needed at micromolar levels to induce FXR (Forman et al., *Cell* 81, 687-695 (1995a)). Fatty acids and farnsoids have been argued as candidate physiological effectors for PPAR and FXR (Keller and Wahli, *Trends Endocrinol. Metab.* 4, 291-296 (1993); Weinberger, *Trends Endocrinol. Metab.* 7, 1-6 (1996)). Although many genes regulated by PPAR are linked to fatty-acid metabolism and fatty acids have been detected in PPAR-inducing chromatographic fractions from human plasma (Banner et al., *J. Lipid Res.* 34, 1583-1593 (1993)), direct interactions of fatty acids with PPARs have not yet been demonstrated.

RXR is a unique member of this orphan receptor family that facilitates many signaling pathways by heterodimerizing with receptors activated by thyroid hormones, retinoids, vitamin D, fatty acids, and farnesoids (Manglesdorf and Evans, Cell 83, 841-850 (1995)). RXR partners also include the orphan receptors COUP (Kliewer et al., *Proc. Natl. Acad. Sci USA* 89, 1448-1452 (1992)), NGF1b/nurr1 (Forman et al., *Cell* 81, 541-550 (1995c); Perhnann and Jansson, *Genes Dev.* 9, 769-782 (1995), and UR/LXR family members (Song et al., *Proc. Natl. Acad. Sci. USA* 91, 10809-10813 (1994); Teboul et al., *Proc. Natl. Acad. Sci. USA* 92, 2096-2100

(1995); Willy et al., *Genes Dev.* 9, 1033-1045 (1995)). The variety of these interactions suggests that RXR performs a key regulatory role in cell physiology.

Surveys of chemical compounds revealed *all-trans*-retinoic acid (ATRA) as an RXR inducer (Manglesdorf et al., *Nature* 345, 224-229 (1990)). However, ATRA did not bind RXR with high affinity, supraphysiological levels were required for activity, and receptors for retinoic acid (RAR) had already been identified (Giguere et al., *Nature* 330, 624-629 (1987); Petkovich et al., *Nature* 330, 444-450 (1987)). Thus, it was proposed that ATRA might be metabolized to a more active form (Manglesdorf et al., *Nature* 345, 224-229 (1990)). Indeed, ATRA isomerizes to 9-*cis*-retinoic acid (9cRA), which activates RXR with a greater potency (Heyman et al., *Cell* 68, 397-406 (1992); Levin et al., *Nature* 355, 359-361 (1992), but activation of RXR and RAR by 9cRA limits its physiological specificity (Allegretto et al., *J. Biol. Chem.* 268, 26625-26633 (1993)). Identification of RXR-specific synthetic "retinoids" and methoprene acid (Lehmann et al., *Science* 258, 1944-1946 (1992); Boehm et al., J. Med. Chem. 37, 408-414 (1994) and J. Med. Chem. 38, 3146-3155 (1995); Harmon et al., *Proc. Natl. Acad. Sci. USA* 92, 6157-6160 (1995), coupled with an inability to detect 9cRA in rat serum (Kojima et al., *J. Biol. Chem.* 269, 32700-32707 (1994)), may argue for the existence of other endogenous RXR-selective terpenoids.

Receptor specificity is one measure of the physiologic importance of a ligand, but matching the ligand potency with its abundance in biological tissues is equally critical. That is, the intracellular concentrations of ligands must be within the ranges of their receptor binding affinities and activation potencies. For example, T_3 and T_4 circulate in human plasma at 2 and 100 nM, respectively (Scully et al., N. Eng. J. Med. 302, 37-48 (1980)), but T_4 activates the thyroid hormone receptor with a 50-fold reduced potency,

as compared with T_3 (Shih et al., *Mol. Endocrinol.* 5, 300-309 (1991)). Consequently, a low-affinity receptor ligand such as T_4 should not be dismissed as nonphysiological on the basis of potency alone, provided that its total effector activity is similar to that of a less abundant but more active one like T_3.

Circulating levels of steroid and thyroid hormones, retinoids, and vitamin D in animal sera are within the ranges of their receptor-activation potencies. Therefore, orphan receptor activators have been sought from biological tissues such as plasma (Shih et al., *Mol. Endocrinol.* 5, 300-309 (1991); Banner et al., *J. Lipid Res.* 34, 1583-1591 (1993)) as well as by a survey of chemical compounds. Chromatographic separation methods can be used to compare the candidate chemically synthesized receptor activators with those extracted from biological tissues to assess their physiological significance. Here we report that a search for RXR activators from bovine serum only revealed compounds chromatographically distinct from 9cRA. These phytol metabolites are considered to be physiological RXR effectors, because they satisfy the above criteria for molecules circulating at concentrations potentially relevant for RXR binding and activation *in vivo*.

SUMMARY OF THE INVENTION

The present invention resides in part in compositions comprising at least one of phytanic acid, derivatives of phytanic acid, phytenic acid and derivatives of phytenic acid. This group of compounds of the invention, that is, phytanic acid and its derivatives and phytenic acid and its derivatives, are collectively called herein "vitamin F". Thus, "vitamin F" consists of one or more of phytanic acid, derivatives of phytanic acid, phytenic acid and derivatives of phytenic acid.

Exemplary derivatives of phytanic acid and phytenic acid are hydroxy-phytanic acid, especially 2-hydroxy-phytanic acid, and hydroxy-phytenic acid, especially 2-hydroxy-phytenic acid. Additional examples are esters and amides of the acids and hydroxy-acids. Preferred examples are carboxylic acid esters, particularly hydrocarbon esters, phospholipid esters and triacylglyceryl esters. Of the hydrocarbon esters, long chain n-alkyl esters are preferred. A hydrocarbon esterifying group preferably contains from 1 to 18 carbon atoms, more preferably 3 to 18 carbon atoms. Preferred phospholipid and triacylglyceryl esters are those of phospholipids and triacylglycerols normally circulating in mammalian serum.

The compositions of the invention can be formulated either as dietary supplements for administration to a mammal, including a human, or as an ingredient of a medium suitable for *in vitro* culture of mammalian cells, especially a serum-free medium.

Accordingly, it is one object of the invention to provide a composition containing vitamin F together with a pharmaceutically acceptable carrier, diluent or builder, especially a carrier suitable for pressing into a tablet.

It is another object of the invention to provide a medium for *in vitro* culture of mammalian cells that contains vitamin F. In one embodiment of this aspect of the invention, the vitamin F is provided as a sterile, concentrated stock solution for the addition to a separately formulated medium. In a preferred embodiment of this aspect of the invention, the vitamin F is formulated as a complex with a protein that transports fatty acids, such as serum albumin. The medium will preferably contain the vitamin F at a final concentration ranging from 1 to 100 μM, preferably 2 to 70 μM, more preferably 2 to 60 μM, most preferably 2 to 10 μM.

It is another object of the invention to provide a method for *in vitro* culture of mammalian cells that comprises growing mammalian cells in a serum-free medium containing vitamin F.

It is yet another object of the invention to provide a method for treating vitamin F deficiency in a mammalian subject, preferably a human, comprising administering to a mammal suffering from vitamin F deficiency a pharmaceutical composition comprising vitamin F. In this embodiment of the invention, the amount of vitamin F in the composition will be one which provides a total plasma concentration of vitamin F of 1 to 100 μM, preferably 2 to 70 μM, more preferably 2 to 60 μM, most preferably 2 to 10 μM. In a preferred embodiment of this aspect of the invention, the vitamin F is formulated as a complex with a serum protein that serves to transport fatty acids, such as serum albumin.

In all of the embodiments of the invention, the vitamin F preferably consists of phytanic acid and/or a derivative of phytanic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Identification of an RXR effector activity from bovine serum. Figure 1 shows the RXR effector activity profile from a chloroform extract of FBS fractionated by reverse-phase HPLC. The chloroform fraction from a Bligh and Dyer extract of 20 ml of FBS was separated by reverse-phase HPLC methods, as described in MATERIALS AND METHODS. Two-minute fractions were pooled and tested for RXR effector activity with the cotransfection assay. Subconfluent (30%) CHO cells were transfected with 1.25 μg of SV-$(DR4)_3$-CAT reporter plasmid (Umesono et al., *Cell* 65, 1255-1266 (1991)), 0.25 μg of human pRS-OR6 or 0.25 μg of CMX-mouse RXRα, 1.25 μg of pCH111, and 0.5 μg pGEM4 DNAs. The pCH111 plasmid (Yao et al., *Nature* 366, 476-479 (1993)) expressing β-galactosidase was included to

correct for differences in transfection efficiency. Normalized CAT activity was plotted against column fraction assayed. A 9cRA standard had a retention time of 7 minutes via this method. The experiment was performed three times with similar results. Note that the coefficient of variation for CAT activity measurements is typically <15%.

Figures 2A and 2B. RXR and RAR activators in bovine serum can be extracted by saponification and ether extraction. Bovine serum (10 ml) was saponified (2 M KOH, heated at 70°C for 30 min) and extracted with diethyl ether. The aqueous phase was acidified, and ether was extracted again. Basic and acidic ether extracts were dried and fractionated separately by reverse-phase HPLC, as described in MATERIALS AND METHODS.

Fig. 2A shows separation of RXR activators from bovine serum by reverse-phase HPLC. One-minute fractions were collected and dried, and a portion (5%) was taken up in a medium containing 5% charcoal-adsorbed FBS for testing by a cis-trans assay, as described for Figure 1.

Fig. 2B shows characterization of RAR activators from bovine serum by reverse-phase HPLC. Three minute fractions were tested by cotransfecting an SV-(βRARE)$_2$-CAT reporter plasmid and a plasmid DNA expressing the human RARα receptor into CHO cells, essentially as described in Figure 1.

Symbols: shaded bars, acidic extract; closed bars, basic extract. Fold induction values are relative to control samples containing methanol vehicle. A control sample in Fig. 2B containing 200 nM ATRA showed a 4.9-fold induction by comparison.

Figures 3A and 3B. Fatty acids copurify with RXR effector activity.

Fig. 3A shows a comparison of molecular ion abundances in HPLC samples near the RXR effector

activity peak. The material remaining from the HPLC-separated fractions (22, 23, 25) obtains as described in the legend for Figure 2A was used for electrospray-ionization mass spectrometry in the negative ion continuum mode (see MATERIALS AND METHODS). Molecular ions (m/z) are plotted against their relative abundances in each sample.

Fig. 3B shows electron impact mass spectra of the major component of the RXR-active fraction from bovine serum extract and authentic phytanic acid standard, obtained from GC/MS analyses of the TMS-derivatized samples. Insets show the reconstructed ion chromatograms for m/z 369, the $(M-CH_3)^+$ ion of the TMS derivative of phytanic acid.

Figures 4A-4D. Phytanic acid is the RXR activator from bovine serum.

Fig. 4A shows RXR activation by fatty acids. Various fatty acids were tested for RXR-specific activity by using the CRBPII-CAT reporter plasmid DNA (Manglesdorf et al., *Cell* 66, 555-561 (1991) and mouse RXRα (Manglesdorf et al., *Genes Dev.* 6, 329-344 (1992)). Arachidonic, farnesoic, linoleic, oleic, palmitic, and phytanic acids (40 μM; Sigma) were added to CHO cells, transfected as described in the legend to Figure 1. CAT activities are the averages from duplicate transfected plates.

Fig. 4B shows phytanic acid dose-response curves. Phytanic acid dilutions were added to CHO cells transfected with either SV-$(DR4)_3$-CAT [DR4] or SV-(CRBPII)-CAT [CRBPII] reporter plasmids in the absence or presence of mouse RXRα. Open squares: CRBPII, no RXR; open circles: CRBPIII +RXR; closed squares: DR4, no RXR; closed circles: DR4,+ RXR. Assays were performed as described in the legend to Figure 1. Results are expressed as averages from duplicate plate lysates.

Fig. 4C shows reaction time for phytanic acid and 9cRA standards separated by reverse-phase HPLC. One

micromole of a phytanic acid standard (Sigma) and 20 nmol of synthetic phytenic acid were injected separately into a sample loop and fractionated as described in MATERIALS AND METHODS. Absorbance was monitored at 226 nm (solid lines I) with a Beckman diode array detector module 168. One nanomole of 9cRA was separately injected, and absorbance was monitored at 325 nm (dotted line). Note that the delay time between absorbance measurement and fraction collection is about 30 seconds. Measured retention times: 9cRA (7.5 min); phytenic acid (20.5 min;, phytanic acid (22.3 min).

Fig. 4D shows RXR effector activity and phytanic acid co-elute by silica gel chromatography. FBS (500 ml) was extracted by the method of Bligh and Dyer (*Can. J. Biochem. Physiol.* 37, 911-917 (1959)). The chloroform-soluble fraction was applied to a silica gel column (40 μM size, 4 x 10 cm bed volume) in 2 ml. The column was developed with 500 ml of 20% ethyl acetate in 80% hexane. Eight-milliliter fractions were collected, and the dried material from two adjacent fractions was taken up in methanol; 5% was tested as described in the legend to Figure 1. The relative fold induction of CAT activity for each sample compared with control methanol vehicle (solid bars) is presented. An authentic phytanic acid standard (5 mg; Sigma) subsequently was separated, and its absorbance (open circles) was monitored at 220 nm. The experiment was repeated, and a similar profile was obtained.

Figure 5 shows the detection of phytenic acid in bovine serum extracts. FBS (40 ml) was extracted by the method of Bligh and Dyer (1959) (see MATERIALS AND METHODS). The dried chloroform extract was dissolved in 400 μl of methanol and separated into 0.3-min fractions by the reverse-phase HPLC conditions described in the MATERIALS AND METHODS. Two plates of CV-1 cells (700,000 cells per microtiter plate) were transfected with either TK-(CRBPII)-LUC reporter and human RXRα

receptor (closed circles) or TK-$(UAS_G)_4$-LUC reporter and GAL4-hRXRα chimeric receptor (open circles) plasmid DNAs by liposome-mediated transfer, as described in MATERIALS AND METHODS. Each plate was incubated with one-half of the fractionated extracts for 40 hrs. Portions of the cell lysates were used to assay luciferase, β-galactosidase, and cytotoxic activities as described (Berger et al., *J. Steroid Biochem. Mol. Biol.* 41, 733-738 (1992)). Single-point luciferase measurements were performed, which typically exhibit 10% variations in this assay. Fold induction is expressed as the relative luciferase activity in the presence of fractionated extracts as compared with untreated cells. The cytotoxicity in two fractions at 21 min is denoted by zero induction. Duplicate control wells to which the FXR activator JH III (40 μM) was added showed onefold inductions; those to which the RXR-specific ligand LG69 (100 nM; Boehm et al., 1995) was added produced 25-fold and 110-fold inductions by using CRBPII-CAT or GAL4-CAT reporters, respectively. Elution positions for phytenic acid and phytanic acid (corresponding to absorbance peaks measured at 220 nm) were R_t = 18.2 and 20.8 min, respectively, and are denoted by arrows.

Figures 6A-6B. Various chlorophyll metabolites activate RXR. Fig. 6A shows the metabolic pathway from chlorophyll to pristanic acid. First, the phytol ester is hydrolyzed, which is followed by oxidation to phytenic acid. Phytenic acid is then hydrogenated to phytanic acid and α-hydroxylation; oxidation leads to pristanic acid. Pristanic acid is finally metabolized by fatty acid O-oxidation pathways. The *trans* isomers of phytol and phytenic acid are illustrated here. Fig. 6B shows the RXR effector activity induced by phytol metabolites and 9cRA. Increasing amounts of 9cRA, phytanic acid, phytenic acid (40% cis/60% trans isomer mixture), and pristanic acid were added to cells transfected with the RXR-specific CRBPII-CAT reporter

plasmid and mouse RXRα. CAT activity was measured from duplicate wells in an assay configured similarly to that described in the legend to Figure 1. Average values for CAT activity from duplicate transfected plates are plotted against increasing activator concentrations. Symbols: circles, 9cRA; diamonds, phytenic acid; squares, phytanic acid; triangles, pristanic acid.

Figure 7 shows the synthesis of 2-hydroxy-phytanic acid. Compound 2: To the solution of phytol 1 (523 ul, 1.5 mmol) in THF (0.50 ml) at 0°C, was added the BMS-THF solution (2.0M, 1.5ml, 2eq) slowly. After the addition, the ice bath was removed and the mixture was stirred at 25°C for 5 hrs. Then the solution was heated to reflux for 1 hour to ensure complete hydroboration. The reaction was quenched with EtOH (1.0 ml) at 0°C followed by 3N NaOH aqueous solution (330 μl). H_2O and sat. NaCl solution, then dried over Na_2SO_4. Concentration and chromatography gave the 1,2-diol 2 as a colorless oil (277 mg, 50%, R_f=0.20 using hexane: EtOAc=10:3). ^{1}HNMR: δ 8.97(br s, 2H), 3.61(m, 1H), 3.44-3.50(m, 2H), 1.50(m, 2H), 1.05-1.34(m, 20H), 0.81-0.85(m, 15H); ^{13}C NMR: δ 76.22, 75.70, 65.08, 64.48 (all for carbons connected to hydroxy group).

Compound 3: To the solution of 1,2-diol 2 (222 mg, 0.71 mmol) in EtOH (10 ml) and H_2O (0.7 ml) at 25°C, was added NaOH (12 mg, 0.30 mmol) and $NaIO_4$ (378 mg, 1.77 mmol, 2.5 eq). The suspension was refluxed for 30 min. and TLC showed the starting material had almost disappeared. The mixture was diluted with ether which was then washed with H_2O, sat. NaCl solution and dried over Na_2SO_4. Concentration gave the aldehyde 3 as a colorless oil (200 mg, 100%, R_f=0.67 using hexane:EtOAc=10:1). ^{1}H NMR: δ 9.55(d, 1H), 2.27(m, 1H), 1.63(m, 1H), 1.46(m, 1H), 1.02-1.33(m, 19H), 0.78-0.82(m, 15H); ^{13}C NMR: δ 204.92 (for carbonyl group).

Compound 4: To the suspension of aldehyde 3 (205

mg, 0.71 mmol) and NH_4Cl (56.7 mg, 1.1 mmol, 1.5 eq) in 1,4-dioxane (1.6 ml) and H_2O (0.4 ml), KCN (60.8 mg, 0.93 mmol, 1.3 eq) was added slowly at room temperature. The mixture was stirred at 25°C for one day. The solution was diluted with ether and washed with H_2O, sat. NaCl solution and dried over Na_2SO_4. Concentration gave the 2-hydroxy nitrile 4 as a colorless oil (205 mg, 92%, R_f=0.24 using hexane:EtOAc=10:1). ^1H NMR: δ 4.37(m, 1H), 2.71(s, 1H), 1.87(m, 1H), 1.53(m, 2H), 1.06-1.36(m, 19H), 0.83-0.87(m, 15H); ^{13}C NMR: δ 119.41, 119.04 (both for cyano group).

Compound 5: A mixture of 2-hydroxy nitrile 4 (85 mg, 0.27 mmol) and 3.6 ml of 37% HCl solution was stirred at room temperature for 5 hrs. and then was refluxed for 12 hrs. The mixture was extracted with ether three times. The combined ether phase was washed with H_2O, sat. NaCl solution and dried over $MgSO_4$. Concentration and chromatography gave the product 5 2-hydroxy-phytanic acid as a colorless oil (63 mg, 70%, R_f=0.27 using CH_2Cl_2:MeOH=10:1). ^1H NMR: δ 4.26(s, 0.5H), 4.17(d, 0.5H), 1.96(s, 1H), 1.50(m, 2H), 1.01-1.38(m, 21H), 0.83-0.87(m, 15H); ^{13}C NMR: δ 179.82, 179.40 (both for carbonyl group); IR (film, $^{-1}$cm): 3447, 2954, 2926, 2868, 1724, 1462, 1377, 1259, 1139; MS calcd. for $C_{20}H_{40}O_3$: 328, found 327(M-H^+).

Figure 8 shows RXR-dependent stimulation of CRBPII-CAT by phytanic acid and 2-hydroxy-phytanic acid. The experiment was performed substantially as described in the legend to Figure 4B.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used: ATRA, *all-trans* retinoic acid; FBS, fetal bovine serum; 9cRA, 9-cis retinoic acid; RAR, retinoic acid receptors.

The present invention resides in part in a vitamin supplement formulation. Such formulations can be prepared in the manner similar to those prepared for other fat soluble vitamins, such as vitamins A, D, E and K. For example, vitamin F or a derivative thereof can be formulated into typical multivitamin or multivitamin/mineral supplements in either liquid or tablet form for oral administration. In particular, it is expected that formulations appropriate for Vitamin A and other retinoid compounds and formulations appropriate for fatty acids would suffice as formulations appropriate for vitamin F. Formulation of fatty acid compounds and retinoids is described, for example, in Chapter 65, pp. 1106 ff. of Remington: The Science and Practice of Pharmacy, 19th ed., copyright 1995 by the Philadelphia College of Pharmacy and Science. Such vitamin supplements containing vitamin F could be administered to subjects suffering from a pathological condition due to deficiency of vitamin F due to dietary deficiency or metabolic impairment.

An exemplary "Vitamin F deficiency" is a condition of having a level of vitamin F in the blood plasma of less than 2 μM including both free and protein bound vitamin F. It is also contemplated that derivatives of phytanic acid, phytenic acid and the 2-hydroxy acids can be used in formulations of the invention. Especially preferred derivatives are esters of the acids. For example, the practitioner should note that vitamin A is

commonly administered as its palmitic acid ester (retinol palmitate is added to milk) or as its acetate ester. Accordingly, long chain and short chain alkyl esters of vitamin F and its derivatives are considered to be preferred compounds which can be used in formulations of the present invention.

As explained above, phytanic acid, phytenic acid and the 2-hydroxy derivatives are expected to be taken up, transported and metabolized in a manner similar to linoleic acid and linolenic acids. Thus, formulation methods for these essential fatty acids can be applied to the formulation of vitamin F compositions.

Phytenic acid can be synthesized using phytol as a precursor compound. The method is similar to that previously described for the synthesis of farnesoic acid and methyl farnesoate (E.J. Cori et al., *J. Am. Chem. Soc.* 90, 5616-5617 (1968)). Briefly, commercially available phytol is oxidized to phytal with activated manganese dioxide. The aldehyde is then converted to the cyanohydrin which is further oxidized to methyl phytenate by manganese dioxide. Phytenic acid is prepared from the ester by saponification with potassium hydroxide in aqueous methanol. Phytanic acid is commercially available from the Sigma Chemical Company, St. Louis, Mo. Fig. 7 shows the synthesis of 2-hydroxy-phytanic acid.

Methyl phytenate shows activity as an activator of RXR, having an ED_{50} about one-half that observed for phytanic acid when tested in the standard cis-trans assay using CRBII-CAT and mouse RXRα (see, Figure 5). Phytenic acid shows an activity profile similar to that of phytanic acid.

In addition to utility as dietary vitamin supplements, vitamin F formulations according to the present invention can be used in *in vitro* tissue and organ culture. Vitamin F and/or its derivatives might be added directly to media used for *in vitro* culture of

cells, tissue sections or grafts or cells or tissue propagated for differentiation into organs.

In determining the amount of vitamin F to be incorporated into compositions for administration to a mammal and the dosage required for the purpose of maintaining normal levels of vitamin F or treating a deficiency of vitamin F, one can determine the pharmacokinetics and bioavailability of the vitamin F by methods commonly known in the art.

Phytanic acid is obtained only from dietary sources and is rapidly oxidized just like other fatty acids, but its specific nutritional requirement is unknown. Abundant sources of phytanic acid in human diets are milk, cheese, and especially butter (Lough, *Lipids* 12, 115-119 (1977)). The caloric value of phytanic acid is only fractionally that of linoleic acid because of their abundance differences, and thus its contribution to cellular energy reserves must be low. Potential pathological signs of vitamin F deficiency could overlap those produced by deficiencies of linoleic-acid, thyroid hormones, vitamins A and D, or other ligands whose receptors cooperate with RXR.

It may be of interest to note that linoleic acid deficiency retards animal growth and that butter efficiently restores the weight lost in rats given fat-free diets. (Burr and Burr, *J. Biol. Chem.* 86, 587-621 (1930); Aaes-Jorgensen, *Physiol. Rev.* 41, 1-41 (1961)). Although linoleic acid has been shown to be one active component, phytanic acid represents another of the growth-promoting substances postulated by Evans and Burr (also fortuitously called "vitamin F" (Evans and Burr, *Proc. Soc. Exp. Biol. Med.* 25, 390-397 (1928)). Phytanic acid can also serve as a growth factor for cells in culture, because linoleic acid replacement of serum albumin and its bound fatty acids has been shown to increase their plating efficiency in serum-free media (Ham, *Science* 140, 802-803 (1963)).

The diterpenoid structure of phytanic acid (Sonneveld et al., *J. Lipid Res.* 3, 351-355 (1962); Lough, *Biochem. J.* 91, 584-588 (1964) suggested that it might be synthesized from mevalonate, but neither endogenous biosynthetic routes nor intestinal microbes contribute to circulating pools in mammals (Steinberg, *Biochem. Biophys. Res. Commun.* 19, 783-789 (1965) and *J. Clin. Invest.* 46, 313-322 (1967)). Phytol metabolites in animal tissues are exclusively derived from the phytyl side chain of chlorophyll. Phytanic acid may be elevated 50-fold and constitute >20% of the fatty acids in patients with Refsum's disease, an inherited metabolic disorder characterized by an α-hydroxylase gene defect that prevents phytanic acid conversion to pristanic acid (Figure 5A; Steinberg, "Phytanic Acid Storage Disease (Refsum's Disease)" in: The Metabolic Basis of Inherited Disease, ed. J.B. Stanbury et al., pp. 731-747, c. 1983 by New York: McGraw-Hill, (1983)).

MATERIALS AND METHODS

Reagents

Fatty acids and other chemicals for enzyme assays were purchased from Sigma Chemical (St. Louis, MO). Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Cell Culture and Transfections

CHQ K1 cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml). Dextran-coated charcoal was used to adsorb lipids from FBS in extract addition experiments (Samuels et al., *Endocrinology* 105, 80-85 (1979)). Transfection assays were performed with N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)/calcium chloride (Chen and Okayama, *Mol. Cell. Biol.* 7, 2745-2752 (1988)).

Briefly, cells were plated at 30% confluence one day before transfection. DNA (3.5 μg) in 200 μl of the DNA/calcium phosphate coprecipitate mixture was added to cells growing in 2 ml media /well of a 6-well tissue culture plate (Falcon, Oxnard, CA). Cells were incubated for 7-8 hrs. at 37°C and washed twice with phosphate-buffered saline (PBS) before ligand or extract additions in fresh media containing 5% charcoal-adsorbed FBS. Methanol (≤ 2% final concentration in media) was used for dissolving extracts and ligands. Liposome-mediated transfection of DNAs into CV-1 cells was performed with N-N1-(2,3)-dioleoyloxylpropyl-N,N,N-trimethylammonium methyl sulfate (DOTAP) according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Liposomes were removed after 2 hrs., and cells were subsequently treated for 40 hrs. with DMEM-FBS alone or with the indicated compounds. Transfected cells were seeded at 7000 cells per well of a microliter plate. For the GAL-hRXRα plate, 300 ng TK-$(UAS_G)_4$-LUC, 500 ng CMX-β-gal, and 100 ng CMX-GAL4-hRXRα plasmid DNAs were added per 10^5 transfected CV-1 cells. For the TK-(CRBPII)-LUC plate, 300 ng TK-(CRBPII)-LUC, 500 ng CMX-βgal, and CMX-hRXRα were added per 10^5 transfected CV-1 cells.

Enzyme Assays

Transfected cells incubated with extracts or ligands for 24 hrs. were washed with PBS two times and then harvested by scraping into 1.2 ml of isotonic buffer (150 mM NaCl, 40 mM Tris-HCI, pH 8, and 1 mM EDTA). Cells were centrifuged briefly (5000 x *g*, 30 s), and the cell pellets were resuspended in 50 μl of 0.25 M Tris-HCl, pH 8. Cells were then subjected to three freeze/thaw cycles (dry ice-ethanol/37°C) before a final centrifugation step (10,000 x *g*, 3 min). Different volumes of supernatants were used to measure CAT activity (20 μl; Seed and Sheen, *Gene* 67, 271-277

(1988)) or β-galactosidase activity (2 μl; Herbomel et al., *Cell* 39, 653-662 (1984)). Luciferase activity was measured as described in Berger et al., *J. Steroid Biochem. Mol. Biol.* 41, 733-738 (1992)).

Bovine Serum Extraction

FBS (Life Technologies) or serum from bovine blood (freely grazing steer raised on silage at North Carolina State University School of Veterinary Medicine) were extracted with chloroform and methanol solvents (Bligh and Dyer, *Can. J. Biochem. Physiol* 37, 911-917 (1959)). Briefly, 10 ml of serum was mixed with 37.5 ml of chloroform and methanol (2:1) and vigorously shaken for 15 min. The mixture was centrifuged at 2000 x *g* for 20 min. To the supernatant was added 12.5 ml each of water and chloroform to separate the phases. The mixture was centrifuged at 9000 x *g* for 15 min, and the chloroform phase was collected. Alternatively, serum was saponified (2 M KOH at 70°C for 30 min) and twice extracted with diethyl ether. Next, the aqueous solution was acidified with concentrated HCl and then extracted with ether again. Chloroform, methanol, or ether was removed by rotary evaporation under vacuum (Buchi Rotavapor R-124 or Speed Vac SC210A; Savant, Farmingdale, .NY).

High Performance Liquid Chromatography

Pure chemical standards or bovine serum extracts were resuspended in 80% methanol and injected into a 1 ml Rheodyne sample loop connected to a Beckman System Gold high-performance liquid chromatography unit (HPLC). The LC system consisted of an RP18 guard column (15 x 3.2 mm, RP18; Alltech, Deerfield, IL) linked to a separation column (4.6 x 25 cm, Econosphere C18, 5 μ particle size; Alltech) and a Gilson FC 203B fraction collector (Middleton, WI). UV absorbance was monitored with a Beckman diode array detector module 168. The

sample was eluted with an 80% methanol/20% 10 mM ammonium acetate mobile phase for 5 min, after which a linear gradient (80-100% methanol in 10 mM ammonium acetate, 20 min) was applied and held at 100% methanol for 10 min. Fractions were collected, dried, and dissolved in DMEM/F12 containing 5% dextran-coated charcoal-absorbed FBS for measurement of CAT activity in the cis-trans assay.

Silica Gel Chromatography

Pure phytanic acid or a chloroform extract of bovine serum was loaded on a silica gel column (4 cm wide x 10 cm height) and eluted with 20% ethyl acetate/80% hexane. In all, 8-ml fractions were collected in 13 x 100-mm glass test tubes, dried by rotary evaporation, resuspended in media containing charcoal-adsorbed FBS, and tested in the cis-trans assay as described.

Mass Spectroscopy

Gas chromatography/mass spectrometry (GC/MS). The trimethylsilyl (TMS) derivative of serum fraction 23 and the phytanic acid standard were prepared by reacting 5 µl of each sample with 10 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA). Reaction mixtures were heated at 70°C for 15 min. An aliquot (0-5 µl) of the reaction mixture was injected onto a Quadrex methylphenyl 5 capillary GC column (30 x 0.25 mm ID, 0.25 mm film) in a Hewlett-Packard 5880 gas chromatograph equipped with a J&W on-column splitless injector and connected to a Finnigan 700 Ion Trap Detector. Full-scan data were obtained over the mass range 40-650 daltons, at a scan rate of 2 s/scan. We used the following temperature program: initial temperature 40°C (1 min hold); programmed to 300°C at 10°/min; hold at 300°C for 30 min.

Fast atom bombardment. A VG ZAB-4F magnetic sector

instrument was used to obtain fast atom bombardment (FAB) data at an accelerating voltage of 8 kV. An Ion Tech atom gun and xenon atoms were used to bombard the sample. The samples were introduced into the mass spectrometer via a coaxial continuous-flow FAB interface. This interface uses a coaxial arrangement of fused silica capillaries to independently deliver the FAB matrix (glycerol) and the analytes, The instrument was scanned from 1000 to 100 daltons at 5 s/decade to acquire the full-scan negative ion data.

Electrospray/ionization MS. Measurements were made on a Fisons-VG Quattro BQ triple-quadruple mass spectrometer equipped with a pneumatically assisted electrospray ion source operating at atmospheric pressure. The HPLC fractions containing biologically active material and phytanic acid were reconstituted in acetonitrile and mixed with equal volumes of the liquid chromatography mobile phase (80% acetonitrile/20% water containing 1% ammonium hydroxide). Samples were introduced by loop injection into the mobile phase at a flow rate of 8 μl/min, and spectra were acquired in the negative ion continuum-mode scan rate. The mass scale was calibrated with polyethylene glycol with an average molecular weight of 400 atomic mass units (amu). Theoretical isotope distributions were computed with Fisons Instruments Opus software.

Synthesis of Phytenic Acid

Phytenic acid was prepared from phytol (Sigma) by adapting a two-step MnO_2 oxidation procedure (Corey et al., *J. Am. Chem. Soc.* 90, 5616-5617 (1968)). Phytol was oxidized to phytal by using activated MnO_2 (Aldrich Chemical, Milwaukee, WI) to give an ~60:40 mixture of geometric isomers by nuclear magnetic resonance (NMR). The isomers were partially separated by chromatography on silica gel, with 5% ethyl acetate in hexane as eluent. The partially purified phytal isomers were each

further oxidized to the corresponding methyl ester by treatment with MnO_2-NaCN-methanol. Chromatography on silica gel with 2% ethyl acetate in hexane gave the individual isomers. Saponification of the methyl esters by KOH in 70:30 methanol/water yielded the free acids. The major isomer was assigned trans stereochemistry on the basis of comparison of the ^1H NMR chemical shifts of the methyl and methylene groups attached to the double bond. As examples, for the trans methyl ester, the chemical shift of the methyl group is 2.11 parts per million (ppm), and the chemical shift for the methylene group is 2.07 ppm. For the cis isomer, the methyl group is relatively shielded by the carbonyl group (1.84 ppm), and the methylene group is relatively deshielded (2.56 ppm).

Hormone Binding

[^{3}H]-ATRA or [^{3}H]-9cRA binding to baculovirus-expressed RAR(α, β, γ) or RXR(α, β, γ) polypeptides was measured as described previously (Allegretto et al., *J. Biol. Chem.* 268, 26625-26633 (1993)). Receptor genes expressing these recombinant proteins were all of human origin except RXRβ and RXRγ, which were derived from the mouse. The assay buffer consisted of 8% glycerol, 120 mM KCl, 8 mM Tris-HCl, 5 mM CHAPS, 4 mM dithiothreitol, and 0.24 mM phenylmethylsulfonyl fluoride, final pH 7.4 (room temperature). The final volume for binding assays was 250 μl, which contained 10-40 μg of protein extract plus 5 nM of [^{3}H]-ATRA for RARs or 10 nM [^{3}H]-9cRA for RXRs, plus varying concentrations of competing ligands. Incubations were performed at 4°C until equilibrium was achieved. Nonspecific binding is defined as that binding remaining in the presence of 1 μM of the appropriate unlabeled retinoid isomer. At the end of the incubation, 50 μl of 6.25% hydroxylapatite was added in the appropriate wash buffer (100 mM KCl, 10 mM Tris-HCl, and either 5 mM CHAPS [RXRs] or 0.5% Triton X-100

[RARs]) to bind the receptor-ligand complexes. Mixtures were vortexed and incubated at room temperature for 30 min and centrifuged, and the supernatants were removed. Hydroxylapatite pellets were washed two more times with wash buffer, and the receptor-ligand complexes were determined by liquid scintillation counting of the pellets. After correcting for nonspecific binding, IC_{50} values were determined. The IC_{50} value is defined as the concentration of competing ligand required to decrease specific binding by 50%, which is determined graphically from a computer-based log-logit plot of the data (Cheng and Prusoff, *Biochem. Pharmacol.* 22, 3099-3108 (1973)).

The following examples of the invention are intended to be illustrative only. The scope of the invention is limited only by the claims following.

Example 1:

RXR Effector Activity from Bovine Serum

We initially attempted to identify activators from bovine serum (Shih et al., *Mol. Endocrinol.* 5, 300-309 (1991)) for an orphan receptor called OR6, which binds to an AGGTCA direct repeat HRE separated by 4 bp (DR4), but only in the presence of RXR (Umesono et al., *Cell* 65, 1255-1266 (1991)). CHO cells were transfected with a DR4-linked CAT reporter plasmid DNA along with an OR6 expression vector, and CAT activity was measured. A lipid extract of FBS was added (Bligh and Dyer, *Can. J. Biochem. Physiol.* 37, 911-917 (1959)), but this had no effect on CAT activity. Although the extract stimulated activity eightfold when RXR was added, RXR alone showed a similar effect. These results suggested that the bovine serum activator was mediating its effects through RXR.

Therefore, the serum effector was compared with 9cRA, a previously described RXR effector from liver (Heyman et al., *Cell* 68, 397-406 (1992)). The chloroform extract of serum was separated by reverse-phase HPLC, and the eluted fractions were tested for RXR

effector activity. Unexpectedly, the RXR activator had a retention time between 19 and 22 min (Figure 1), which did not coincide with the elution profile for a 9cRA standard (R_t = 7 min). Because 9cRA is chemically similar to ATRA, we added a tracer amount of [^{3}H]-ATRA (1 nM) to a serum sample to determine whether retinoic acid could be extracted by this method. Nearly all of the radioactivity (83%) was found in the chloroform fraction, thus supporting the utility of the Bligh and Dyer method for extracting retinoids.

RXR Effector Activity Is Distinct from 9cRA

Because the retinoic acid in serum may have been resistant to extraction by the Bligh and Dyer method, a procedure specifically used to isolate retinoids was used here to characterize the RXR effector (Kojima et al., *J. Biol. Chem.* 269, 32700-32707 (1994)). We also wanted to exclude vitamin supplements that are sometimes given to donor herds as potential sources for exogenous retinoids. Therefore, serum from a freely grazing steer was saponified and ether extracted, and then the aqueous phase was acidified and extracted with ether again. [^{3}H]-ATRA in a parallel sample was quantitatively extracted by ether (95%) from the acidified aqueous solution, marking this as another effective means for retinoid isolation. In contrast, RXR-inducible CAT activity was found only in the ether extract of the basic solution. This material was separated by the HPLC conditions described above (Figure 1), and the 1-min fractions were collected and tested for RXR effector activity. An RXR-specific activator (R_t = 23-24 min, Figure 2A) was identified that eluted later than ATRA or 9cRA (Rt = 8.8 and 7.5 min, respectively). Therefore, both saponified and nonsaponified serum extracts contained an RXR activator with chromatographic properties distinct from 9cRA.

It was conceivable that retinoids were destroyed by

this rigorous extraction method. Therefore, DNAs for the human retinoic acid receptor (RARα) and βRARE-CAT reporter were transfected into cells to permit detection of the RAR activators ATRA and retinol (Giguere et al., *Nature* 330, 624-629 (1987); Sucov et al., *Proc. Natl. Acad. Sci. USA* 87 5392-5396 (1990)). Activities coincident with 9cRA, ATRA (R_t = 7.5 and 8.8 min), and retinol (R_t = 20 min) were confined to the acidified extract (Figure 2B); none was found in the ether extract of the basic solution in which the RXR effector activity was observed. In addition, a broad range of activity more polar (Rt < 20 min) than retinol was seen. This material may correspond to hydroxylated retinol metabolites, such as 4-oxo-retinol, the acid derivative of which was shown to activate RAR (Pijnappet et al., *Nature* 366, 340-344 (1993)). Nevertheless, although peaks of activity cannot be assigned, it is clear that RAR and RXR activators have distinct pH-dependent partitioning characteristics in ether. Moreover, the functional integrity of RAR activators is maintained during extraction. By inference, 9cRA should have been found in the acidic fraction, but no corresponding RXR effector activity was detected here. These results suggest that the bovine serum activator is distinct from 9cRA, but they do not exclude the possibility that 9cRA may still be an intracellular signal in the liver or kidney, where it was originally described (Heyman et al., *Cell* 68, 397-406 (1992)).

Fatty Acids Copurify with RXR Effector Activity

To characterize the molecular structure of the RXR activator, the active fraction of the basic ether extract (R_t = 23 min) and two adjacent inactive ones (R_t = 22 and 25 min) were analyzed by various mass spectrometric techniques. Negative ion electrospray spectra, obtained by flow-injection analyses of these fractions, contained ions of m/z 283 and 311 (Figure

3A). The abundance of the m/z 311 ion corresponded to the RXR activities in these fractions (Figure 2A), whereas the abundance of the m/z 283 ion did not follow the RXR activities. Relative isotopic abundance measurements for these negative ions predicted the molecular formulas $C_{18}H_{36}O_2$ and $C_{20}H_{40}O_2$ for the molecular weight 284 and 312 Da components, which are consistent with the elemental compositions of stearic acid and phytanic acid, respectively. The same two prominent $(M-H)^-$ ions, m/z 283 and 311, were also observed by negative-ion fast atom bombardment mass spectrometry (our unpublished observations).

GC/MS analysis of the TMS-derivatized saponified sample showed a peak corresponding in mass to the $(M-CH_3)^+$ fragment ion (m/z 369) of the TMS derivative of phytanic acid, as well as a low-abundance peak corresponding to the molecular ion (m/z 384). The full-scale mass spectrum and the retention time of this component were in agreement with those of the TMS derivative of authentic phytanic acid (Figure 3B), run under identical conditions. Co-chromatography of the sample and the phytanic acid standard gave a single peak in the reconstructed ion chromatogram for m/z 369, as well as for other characteristic ions.

Phytanic Acid Is the Serum RXR Activator

A single chromatographic step was deemed unlikely to have separated the RXR activators from other serum components. Nonetheless, the above results prompted us to examine a collection of fatty acids for RXR activation. Although linoleic, oleic, stearic, farnesoic, palmitic, and arachidonic acids (40 μM) were without effect, a similar amount of phytanic acid produced a fivefold induction of CAT activity with the RXR-specific CRBPII-CAT reporter (Figure 4A). Phytanic acid responsiveness was RXR dependent, using both DR4-CAT and CRBPII-CAT reporters (Figure 4B). The dynamic range for

phytanic acid activation of mouse RXRα was between 4 and 64 μM, with cellular toxicity exhibited at higher doses. Other compounds with phytyl side chains were tested, including phytol, vitamin E, and vitamin K_1, but these failed to activate RXR when added at 50 μM concentrations.

Absorbance profiles depicting the retention times for phytanic acid, its metabolic precursor phytenic acid, and 9cRA have been included for reference (Figure 4C). Note that the retention time for pure phytanic acid (R_t = 22.5 min) coincides with that of the serum RXR activator (Figure 2A). The time difference between the phytanic acid absorbance and the RXR effector activity is due to a 30 sec delay time in this LC system. As further evidence for identity, a phytanic acid standard coeluted with the serum RXR activator when separated by silica gel chromatography with 20% ethyl acetate in hexane as the developing solvent (Figure 4D). Together these results show that the RXR activator in serum corresponds to phytanic acid.

Detection of Phytenic Acid

The DR4-CAT reporter plasmid was originally selected for isolating RXR activators from bovine serum in favor of CRBPII-CAT because of its more robust induction in CHO cells (Figure 4A). Despite its RXR dependency, DR4 had not been previously described as an RXR-responsive element, and thus its use here might be judged inappropriate. Therefore, we sought to confirm the presence of phytanic acid in bovine serum extracts by using assays designed with greater RXR specificity. Four copies of the DNA binding site (upstream activating sequence UASG) for the yeast GAL4 gene product or a CRBPII response element were separately inserted in the herpes simplex virus thymidine kinase promoter that was linked to the firefly luciferase gene (Forman et al., *Cell* 81, 541-550 (1995)). These reporter plasmids were

independently cotransfected into CV-1 cells with CMX-GAL4-RXR (Forman et al., *Cell* 81, 541-550 (1995)), a chimeric receptor fusing the GAL4 DNA-binding domain to the human RXRα ligand-binding domain, or with CMX-human RXRα (Yao et al., *Nature* 366,476-479 (1993)) as the respective receptor plasmids.

A chloroform extract of FBS (Bligh and Dyer, *Can. J. Biochem. Physiol.* 37, 911-917 (1959)) was separated by reverse-phase HPLC as described in Figure 2B, but this time the eluate was collected in 0.3 min fractions to afford greater analytical resolution. The material was divided in half, each was added to the two sets of CV-1 cells cotransfected as described above, and normalized luciferase activities were measured. The superimposable profiles contained two peaks of activity (19.0 and 21.6 min; Figure 5) corresponding to the absorbance profiles for phytenic acid and phytanic acid, respectively (R_t = 18.2 and 20.8 min for this particular column). The amounts of serum extract used for these assays were about 10-fold greater than those used earlier (Figure 2, A and B). Thus, the cytotoxicity shown in two adjacent fractions (R_t ~21 min) may have been due to increased amounts of stearic acid that elutes just before phytanic acid (Figure 3A). Nevertheless, phytenic acid now became detectable, but as before (Figure 2A), a peak coincident with 9cRA (R_t = 7 min) was not found. Thus, these data confirm and extend the results previously obtained with the DR4-CAT reporter plasmid to identify both phytanic acid and phytenic acid in bovine serum extracts.

Phytol Metabolites as Transcriptional Signals

Phytanic acid and phytenic acid levels in normal human serum are 6 μM and 2 μM, respectively (Avignan, Biochem. Biophys. Acta 166, 391-394 (1966)). Like other fatty acids, 70% of the phytanic acid probably exists as triacylglycerol or phospholipid esters that are rapidly

oxidized and that vary with dietary conditions (Mohrhauer and Holman, *J. Lipid Res.* 4, 151-159 (1963); Mize et al., *J. Lipid Res.* 7, 692-697 (1966) and *J. Clin. Invest.* 48, 1033-1040 (1969)). Although the estimated free phytanic acid (2 μM) is only at the threshold for RXR stimulation (Figure 4B), equipotent phytenic acid may also contribute to the RXR effector pool (Mize et al., *J. Lipid Res.* 7, 692-697 (1966). In addition, the charcoal-treated serum used in this bioassay may have adsorbed some of the added phytanic acid, thereby reducing its effective concentration. Phytol is unlikely to be an RXR effector because at 50 μM it neither bound nor activated RXR, whereas at higher concentrations it showed cytotoxic effects. Finally, the limited effector activity shown by pristanic acid suggests that other phytanic acid metabolites, such as α-hydroxy-phytanic acid or ω-carboxylated derivatives, may also be RXR inducers (Billimoria et al., *Lancet* 1(8265), 194-196 (1982)).

The EC_{50} values for RXR activation by phytol metabolites were estimated assuming that the dose-response maxima were reached at 64 μM (Figure 3A). These nonsaturating dose-response curves are probably due to cellular toxicity in which, above 64 μM, the limits for fatty-acid binding to serum albumin were exceeded (Herndon et al., *J. Clin. Invest.* 48, 1017-1040 (1969); Spector et al., *J. Lipid Res.* 10, 56-67 (1969)). Alternatively, some of the natural isomers of phytanic acid (Baxter and Milne, *Biochim. Biophys. Acta* 176,265-277 (1969)) may inhibit RXR binding. Integration of the effector activities produced by each of these isomers in the tested sample of phytanic acid may thus give rise to the nonsaturable activity profile. Nevertheless, given that their plasma levels approximate their RXR binding affinities and activation potencies, phytanic acid and phytenic acid meet our criteria for humoral RXR effectors.

The units of RXR effector activity caused by phytanic acid were only crudely assessed in our experiments, but the activity caused by the injected serum sample (Figure 2A) can be accounted for by the peak of activity found in fractions 23 and 24. The contribution of phytanic acid to the total serum activity can be estimated on the basis of its concentration (5 mg/100 ml) in bovine plasma (Avignan, *Biochim. Biophys. Acta* 166, 391-394 (1966)). Five percent (0.5 ml) of the 10 ml-extracted serum sample was assayed for RXR effector activity by using the DR4-CAT reporter plasmid (Figure 2A). Thus, the estimated phytanic acid (0.025 mg or 80 nmol) in fractions 23 and 24 (Figure 2A) in 4 ml of media is 20 μM, which approximates the EC_{50} value in the dose-response curve. Importantly, the induction in this experiment was submaximal, evidence for which is given by the threefold increase (Figure 2A) as compared with the 16-fold maximum induction seen in Figures 1 and 4B. Because phytanic acid and phytenic acid constitute the only RXR-inducing molecular species in serum (Figure 5), both together to define the bulk of activity.

Distinct Humoral Diterpenoid Activators for RAR and RXR

Circulating ATRA levels are 6 nM (Napoli et al., *J. Lipid Res.* 26, 387-392 (1985); Tang and Russel, *J. Lipid Res.* 31, 175-182 (1990)), which are sufficient for RAR stimulation (Giguere et al., *Nature* 330, 624-629 (1987)) but not for RXR activation (Manglesdorf et al., *Nature* 345,224-229 (1990); Allegretto et al., *J. Biol. Chem.* 268, 26625-26633 (1993)). In contrast, retinol binds RAR 35 times less potently than ATRA (Repa et al., Proc. Natl. Acad. Sci USA 90, 7293-7297 (1993)), and 1000 times more retinol is required for RAR activity (Giguere et al., *Nature* 330, 624-629 (1987)). Although the circulating retinol in human plasma (2 μM) may seem more than adequate for RAR induction (Miller et al., *Anal.*

Biochem. 138, 340-345 (1984)), its effective concentration may be limited by retinol-binding proteins such as CRBPII (Ong, *Arch. Dermatol.* 123, 1693a-1695a (1987)). The RAR activators from the acidified ether extracts of serum described here may support both ATRA and retinol as circulating effectors (Figure 2B). Activators more polar than retinol could be hydroxylated metabolites such as 14-hydroxy-4,14-*retro*-retinol (Buck et al., *Science* 254, 1654-1656 (1991)) and 13,14-dihydroxy-retinol that would likely have retention times less than that of retinol (Derguini et al., *J. Biol. Chem.* 270, 18875-18880 (1995)). Description of the ATRA metabolites 4-oxo-retinoic acid and 3,4-didehydro-retinoic acid as RAR inducers may also support this hypothesis (Allenby et al., *Proc. Natl. Acad. Sci. USA* 90, 30-34 (1993); Pijnappel et al., *Nature* 366, 340-344 (1993)). Further fractionation of these extracts might help to establish the identities of these RAR activators.

At the same time, our findings seem to diminish support for retinoids as circulating RXR activators in bovine serum. A peak of RXR effector activity corresponding to 9cRA was not detected with our methods, a result that agrees with its reported absence in normal rat serum (Kojima et al., *J. Biol. Chem.* 269, 32700-32707 (1994)). Based on the dose response curve in Figure 6B as well as from others (Heyman et al., *Cell* 68, 397-406 (1992); Pijnappel et al., *Nature* 366, 340-344 (1993)), 50 nM 9cRA should have been detectable with our assay system. Because no peak of RXR effector activity corresponding to 9cRA was observed with a 20-ml extract sample (Figure 5), 9cRA must be present in bovine serum at concentrations lower than 0.5 nM as tested in the 200-μl well.

Example 2:

Phytol Metabolites Bind and Activate RXR

Phytanic acid, phytenic acid and other phytol metabolites derived from the phytyl chain of chlorophyll (Figure 6A) were compared for dose-dependent stimulation of RXR, using CRBPII-CAT as the reporter plasmid. Synthetic phytenic acid, consisting of 40% cis and 60% *trans* isomers, was tested along with phytanic acid, pristanic acid, and 9cRA. The dose responses for RXR activation by phytenic and phytanic acids were similar, having EC_{50} values of 15 μM, whereas pristanic acid, a phytanic acid metabolite, stimulated with a lower potency and efficacy (Figure 6B). By comparison, 9cRA induced activity at a concentration ~200-fold lower than that for either of phytanic acid or phytenic acid. Testing of the separated isomers of phytenic acid at 32 μM revealed that the *trans* isomer induced RXR effector activity 4.5-fold, which paralleled that of phytanic acid, whereas the *cis* isomer was nearly inactive.

Direct interaction of phytanic acid with RXR was measured by displacing [^{3}H]-9cRA bound to baculovirus-expressed RXR proteins with unlabeled 9cRA or phytol and its metabolites (Allegretto et al., *J. Biol. Chem.* 268, 26625-26633 (1993)). Phytenic acid and phytanic acid competed one-half of the [^{3}H]-9cRA binding to human RXRα with a K_i of ~2.3 and 4.4 μM, whereas pristanic acid did so only at 15.1 μM (Table 1). The K_i for phytol was 67.2 μM, suggesting that it is an ineffective 9cRA competitor. Moreover, the binding affinities of phytanic acid and phytenic acid for the individual RXRs were similar. In contrast, phytol metabolites did not displace [^{3}H]-ATRA from RAR even at 100 μM, which demonstrates their binding specificity. Finally, the binding affinity of 9cRA for RXR is 200 times greater than that of phytanic acid or phytenic acid (Table 1), which is similar to their relative potencies for RXR activation (Figure 6B). We conclude that the transcriptional effects of phytol metabolites are specifically transduced by directly binding to RXR.

The invention being thus described, various modifications of the materials and methods used in its practice will be readily apparent to one of ordinary skill in the art. Such modifications are to be considered within the scope of the invention defined by the claims below.

Table 1. Competition of phytol and metabolites for [^{3}H]-ATRA and [^{3}H]-9cRA binding to RXRs

Compound	K_i					
	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
ATRA[a]	18.2 ± 2.1	17.3 ± 1.8	14.6 ± 1.8	10.2 ± 1.5	22.1 ± 2.3	19.8 ± 0.6
9-cis RA[a]						
Phytol[b]	> 100	70 ± 30	> 100	67.2 ± 32.8	41.9 ± 0.2	47.1 ± 12.6
Pristanic acid[b]	> 100	74.8 ± 25.3	88 ± 12	15.1 ± 8.6	13.3 ± 3.3	25.6 ± 17.2
Phytanic acid[b]	> 100	> 100	> 100	4.4 ± 0.7	4.1 ± 0.2	3.6 ± 0.7
Phytenic acid[b]	> 100	> 100	> 100	2.3 ± 0.4	3.7 ± 1.1	2.4 ± 0.4

[a] Values are in nM and represent the mean ± SEM of two determinations.

[b] Values are in μM and represent the mean ± SEM of two determinations, except for phytanic and phytenic acid binding to RXRs, where n = 3.

Binding assays were performed as previously described (Allegretto *et al.*, 1993).

CLAIMS

What is claimed is:

1. A composition comprising vitamin F, wherein said vitamin F consists of at least one of phytanic acid, a derivative of phytanic acid, phytenic acid and a derivative of phytenic acid; and a pharmaceutically acceptable carrier, diluent or builder.

2. A composition according to claim 1, wherein said carrier, diluent or builder is useful for pressing into a tablet.

3. A composition according to claim 1 or 2, wherein said carrier comprises a serum protein.

4. A composition according to any one of claims 1 to 3, wherein said vitamin F is provided as an ester.

5. A composition according to any one of claims 1 to 4, wherein said vitamin F is esterified by a hydrocarbon alcohol.

6. A composition according to any one of claims 1 to 5, wherein said composition is for administration to a mammal and contains an amount of vitamin F sufficient to provide a concentration in the plasma of said mammal of from 1 to 100 μM.

7. A method for treating vitamin F deficiency in a mammal comprising administering a composition of any one of claims 1 to 6 to a mammal suffering from vitamin F deficiency.

8. A method for maintaining vitamin F in the blood plasma of a mammal comprising administering a composition of any one of claims 1 to 6 to a mammal.

9. Use of vitamin F to make a composition for treating a mammal for vitamin F deficiency.

10. Use of vitamin F to make a composition for maintaining blood plasma levels of vitamin F in a mammal.

11. A serum-free medium for the culture of mammalian cells or tissues or organs *in vitro* which comprises vitamin F, wherein said vitamin F consists of at least one of phytanic acid, a derivative of phytanic acid, phytenic acid and a derivative of phytenic acid.

12. A serum-free medium according to claim 9, wherein said vitamin F is complexed to a serum protein.

13. A serum-free medium according claim 9 or 10, wherein said vitamin F is esterified by a hydrocarbon alcohol.

14. A serum free medium according to any one of claims 9 to 11, wherein said vitamin F is present at a concentration ranging from 1 to 100 μM.

15. A method for culturing mammalian cells *in vitro*, which comprises growing said mammalian cells in a serum-free medium according to any one of claims 9 to 12.

FIG. 1

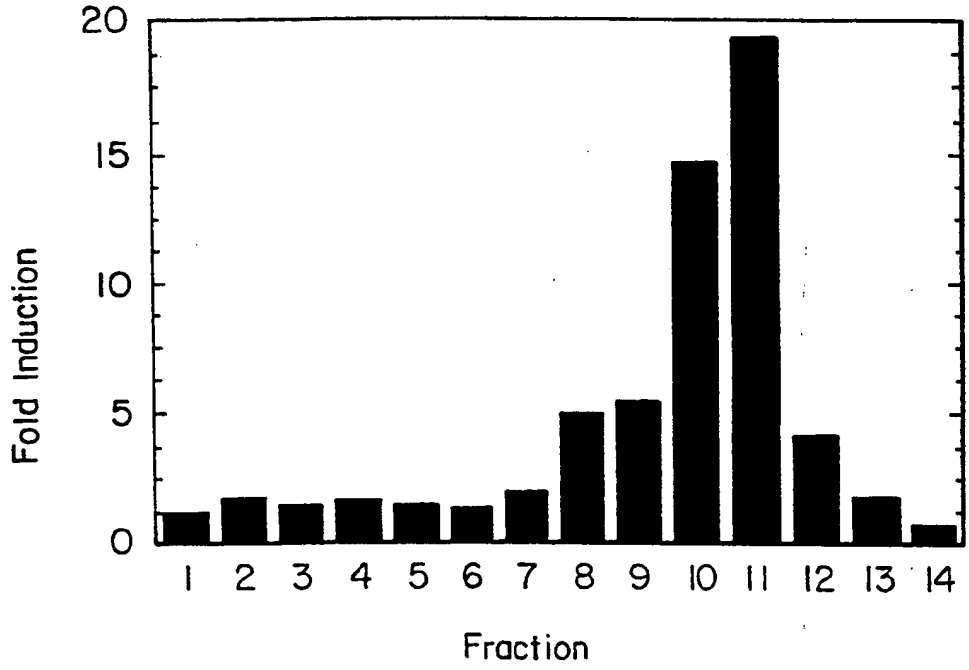


FIG. 2A

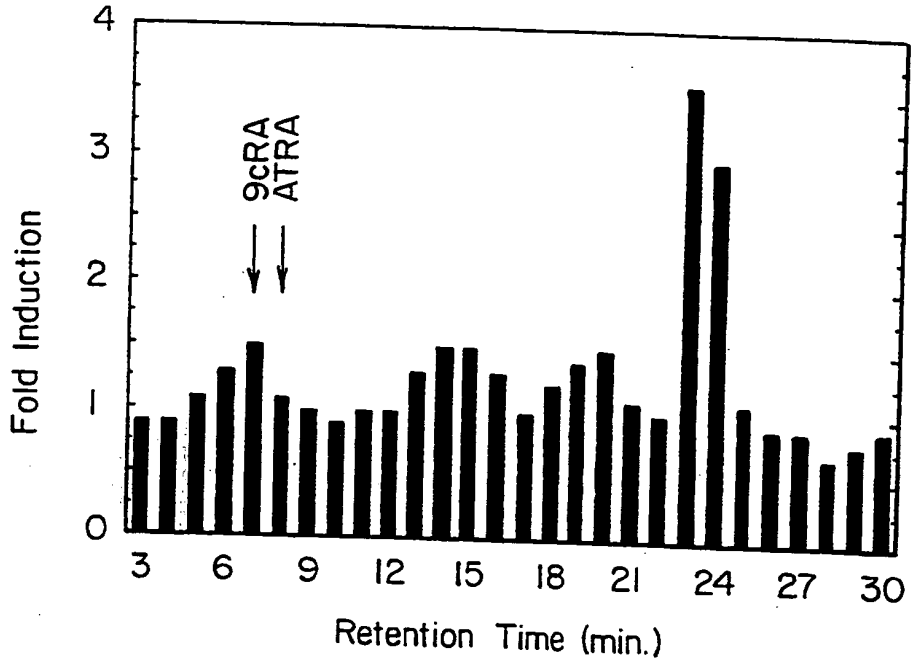

4
3
2
1
0
Fold Induction
9cRA
ATRA
3
6
9
12
15
18
21
24
27
30
Retention Time (min.)

FIG. 2B

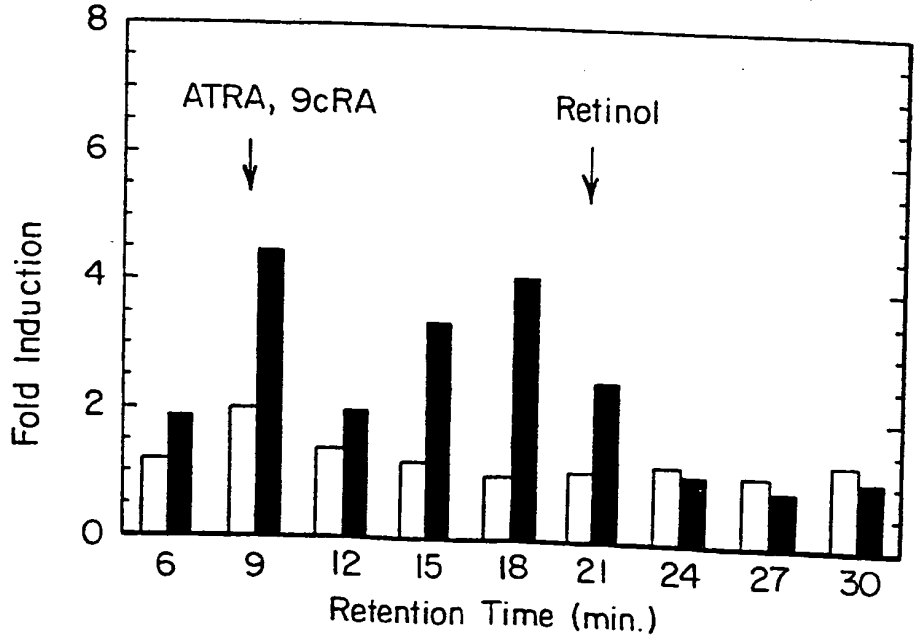
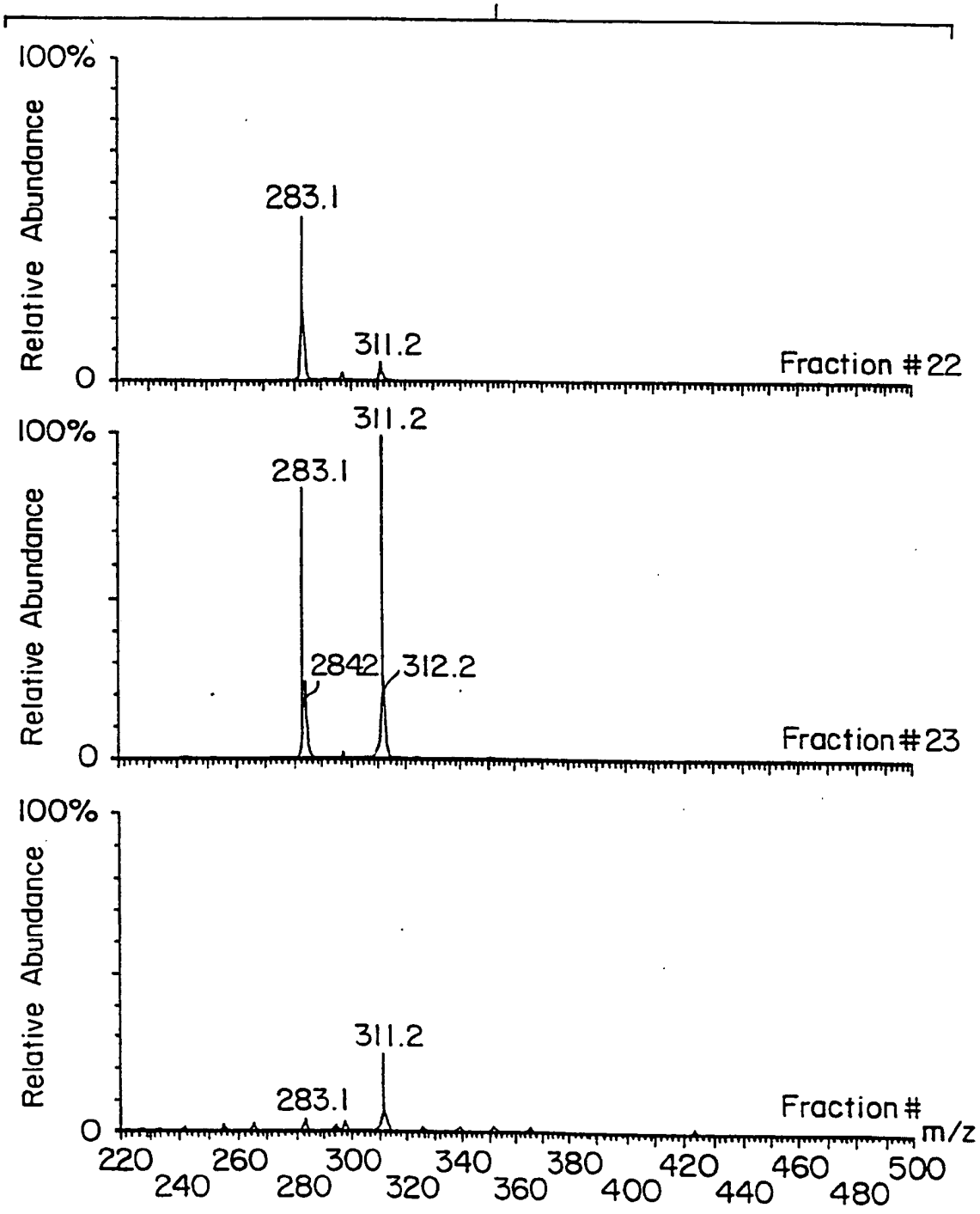

8
6
4
2
0
Fold Induction
ATRA, 9cRA
Retinol
6
9
12
15
18
21
24
27
30
Retention Time (min.)

FIG. 3A


100%
Relative Abundance
283.1
311.2
0
Fraction #22
100%
311.2
283.1
2842
312.2
Relative Abundance
0
Fraction #23
100%
Relative Abundance
311.2
283.1
0
Fraction #
m/z
220 240 260 280 300 320 340 360 380 400 420 440 460 480 500

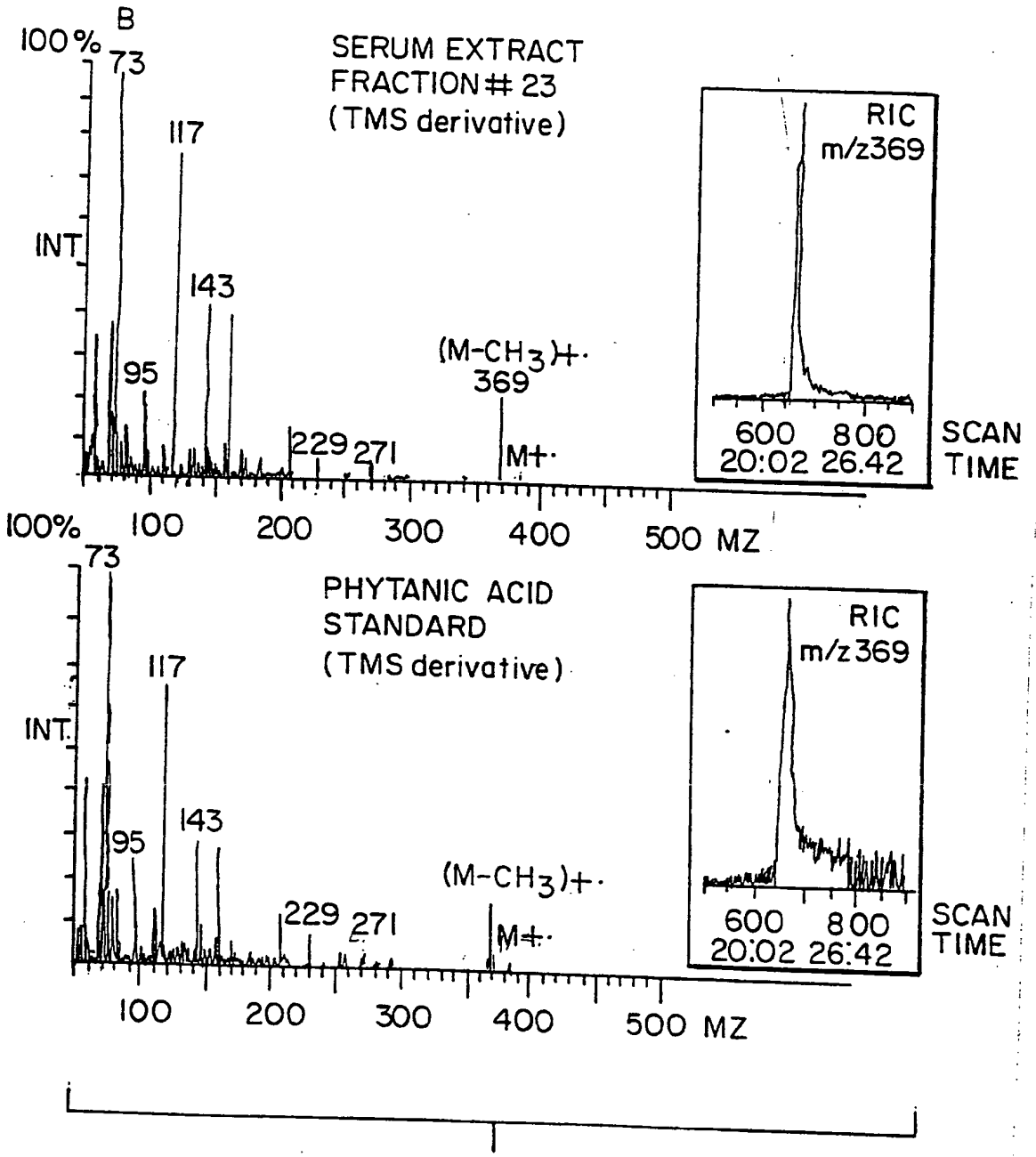

B
100%
73
117
INT.
143
95
(M-CH3)+·
369
229
271
M+·
SERUM EXTRACT
FRACTION # 23
(TMS derivative)
RIC
m/z369
600 800
20:02 26.42
SCAN
TIME
100%
100
200
300
400
500 MZ
73
117
INT.
143
95
(M-CH3)+·
229
271
M+·
PHYTANIC ACID
STANDARD
(TMS derivative)
RIC
m/z369
600 800
20:02 26:42
SCAN
TIME
100
200
300
400
500 MZ

FIG. 3B

FIG. 4A

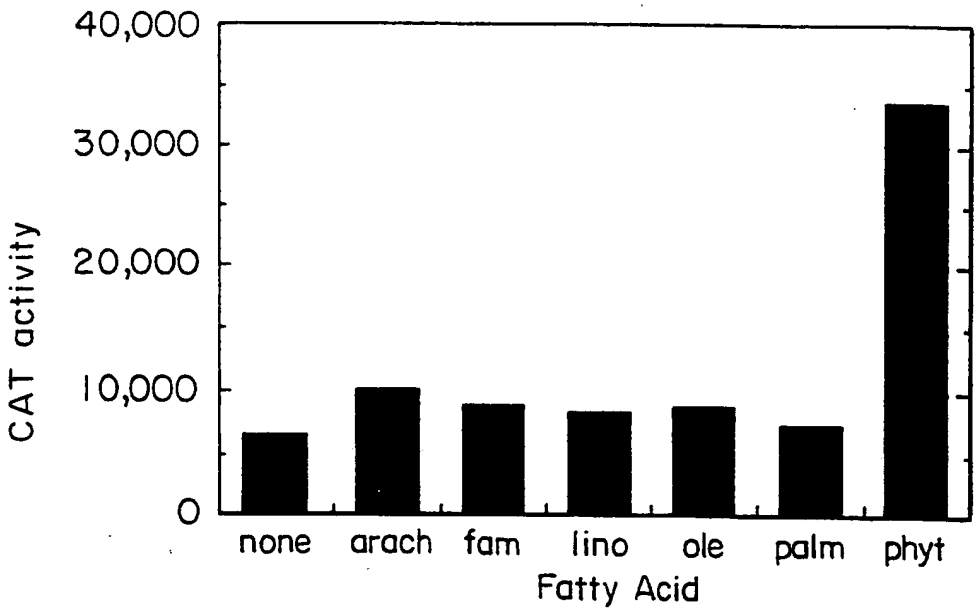

40,000
30,000
20,000
10,000
0
CAT activity
none
arach
fam
lino
ole
palm
phyt
Fatty Acid

FIG. 4B

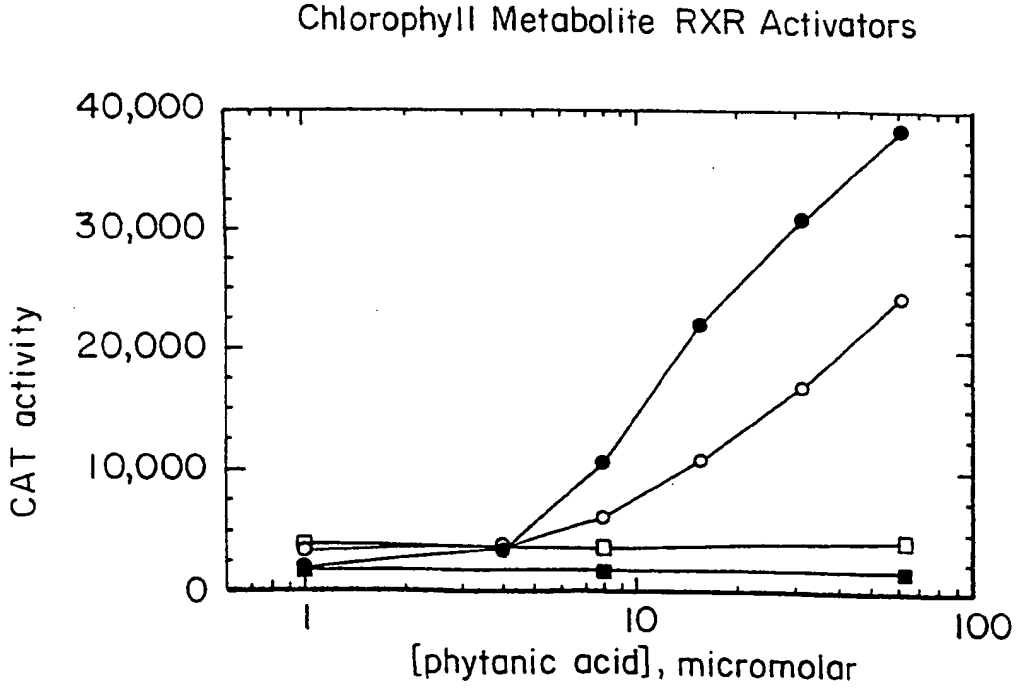

Chlorophyll Metabolite RXR Activators
40,000
30,000
20,000
10,000
0
CAT activity
1
10
100
[phytanic acid], micromolar

FIG. 4C

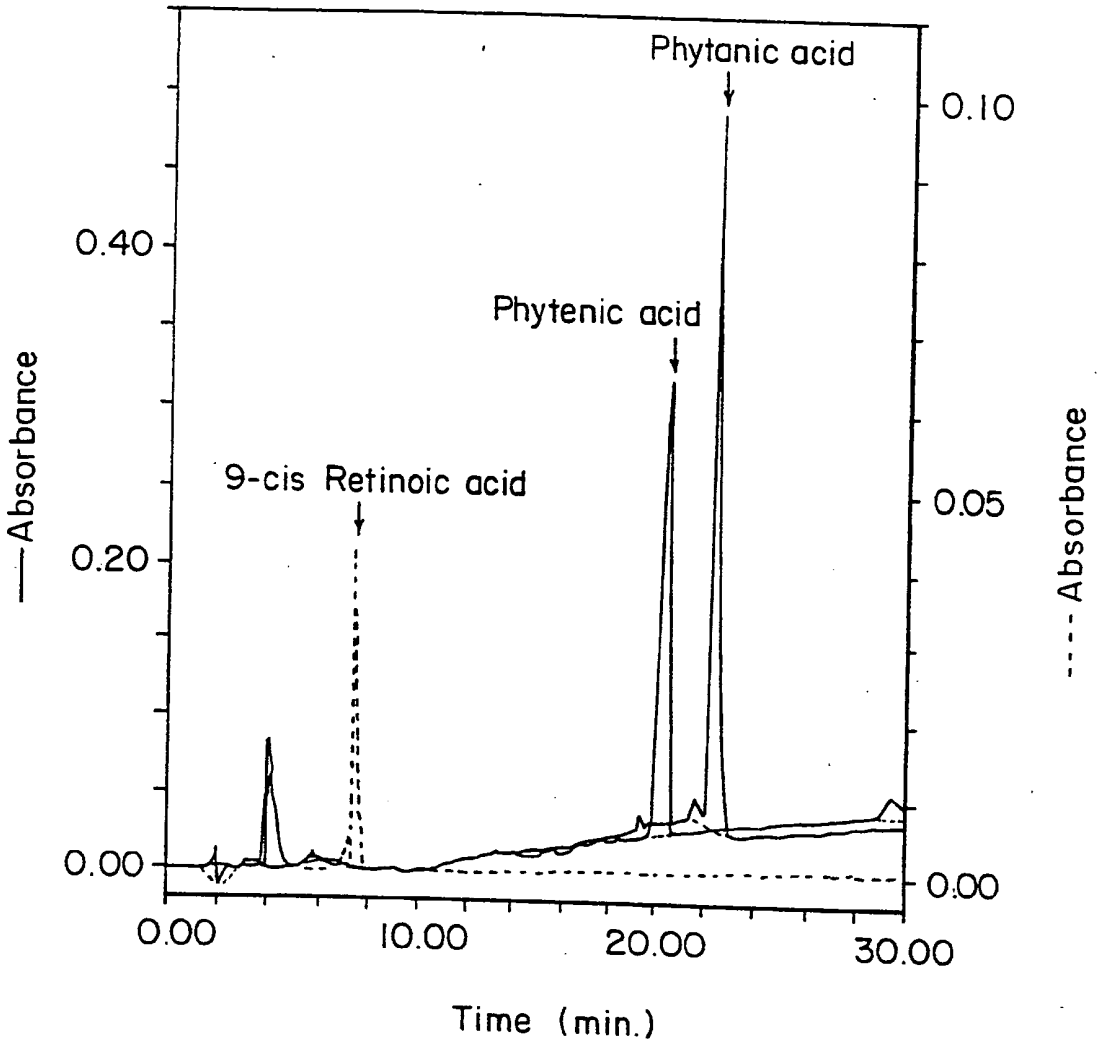
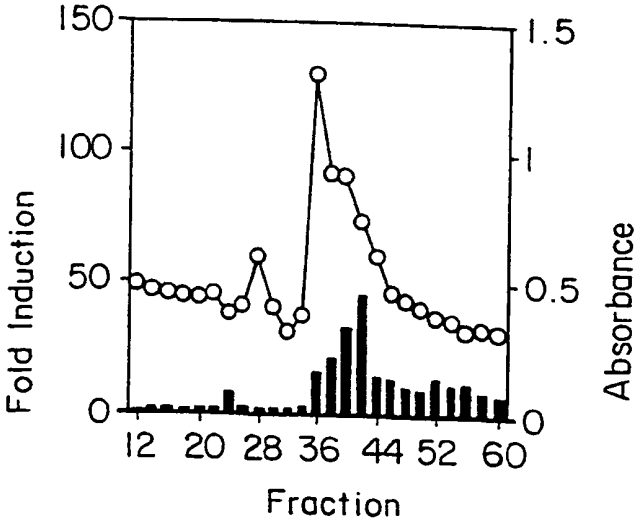

Phytanic acid
Phytenic acid
9-cis Retinoic acid
0.40
0.20
0.00
0.10
0.05
0.00
—Absorbance
---- Absorbance
0.00
10.00
20.00
30.00
Time (min.)

FIG. 4D


150
100
50
0
1.5
1
0.5
0
Fold Induction
Absorbance
12 20 28 36 44 52 60
Fraction

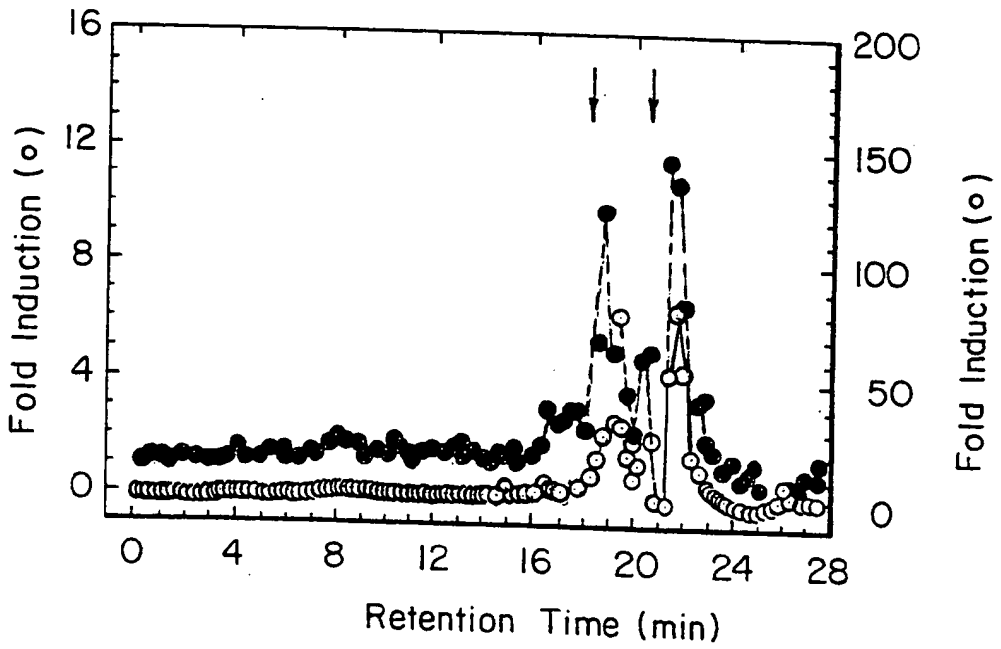

16
12
8
4
0
Fold Induction (o)
200
150
100
50
0
Fold Induction (o)
0
4
8
12
16
20
24
28
Retention Time (min)

FIG. 5

FIG. 6A

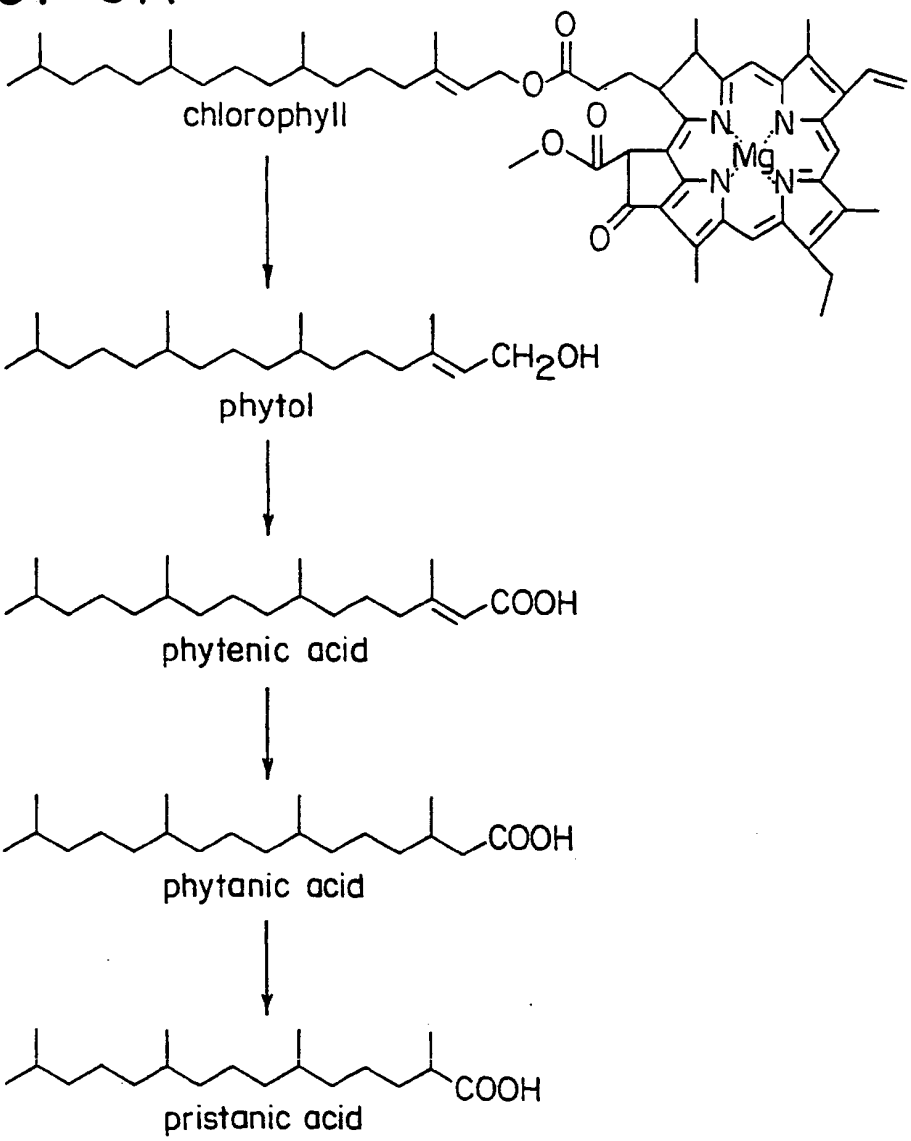
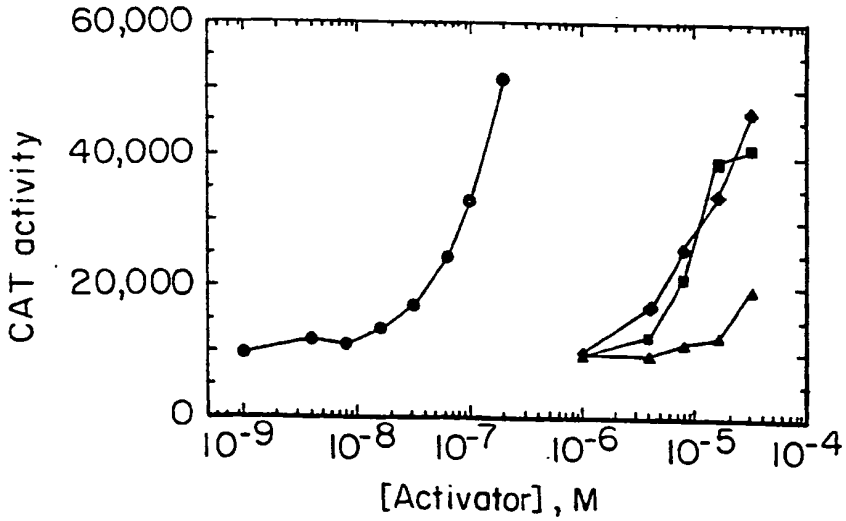

chlorophyll
phytol
CH_2OH
phytenic acid
COOH
phytanic acid
COOH
pristanic acid
COOH
Mg
N
O

FIG. 6B


60,000
40,000
20,000
0
CAT activity
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[Activator], M

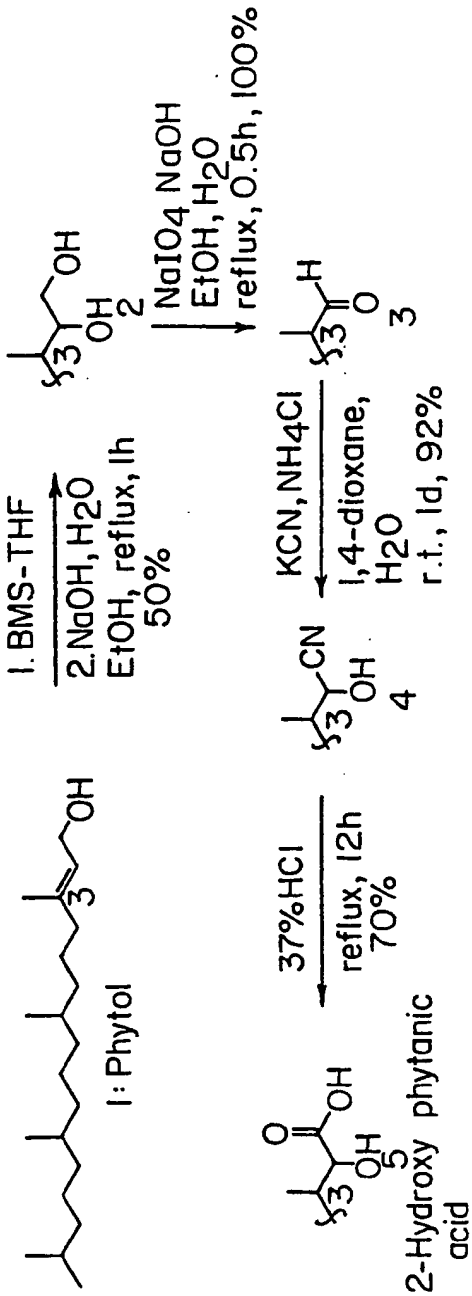

1: Phytol
1. BMS-THF
2. NaOH, H2O
EtOH, reflux, 1h
50%
2
NaIO4 NaOH
EtOH, H2O
reflux, 0.5h, 100%
3
KCN, NH4Cl
1,4-dioxane,
H2O
r.t., 1d, 92%
4
37% HCl
reflux, 12h
70%
5
2-Hydroxy phytanic acid

FIG. 7

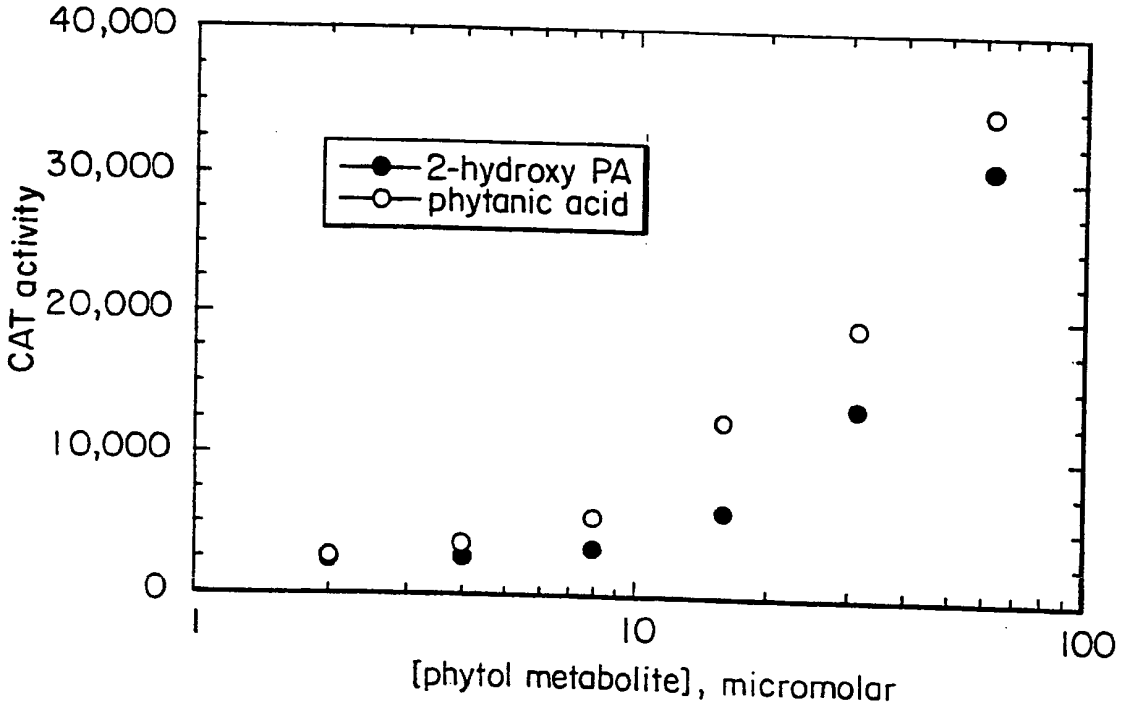

40,000
30,000
20,000
10,000
0
CAT activity
2-hydroxy PA
phytanic acid
1
10
100
[phytol metabolite], micromolar

FIG. 8

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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International Bureau

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(10) International Publication Number
WO 97/09039 A3

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(21) International Application Number: PCT/US96/15205

(22) International Filing Date:
6 September 1996 (06.09.1996)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/003,443 8 September 1995 (08.09.1995) US

(71) Applicant *(for all designated States except US)*: **THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; National Institutes of Health, Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WEINBERGER, Cary** [US/US]; 107 Spring Valley Road, Carrboro, NC 27510 (US). **KITAREEWAN, Sutisak** [US/US]; 312 E. 66th Street, Apt. 2B, New York, NY 10021 (US).

(74) Agents: **MURPHY, Gerald, M., Jr.** et al.; Birch, Stewart, Kolasch & Birch, LLP, P.O. Box 747, Falls Church, VA 22042-0747 (US).

(81) Designated States *(national)*: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN.

(84) Designated States *(regional)*: ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
13 September 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 97/09039 A3

(54) Title: COMPOSITION COMPRISING VITAMIN F

(57) Abstract: RXR is a nuclear receptor that plays a central role in cell signaling by pairing with a host of other receptors. Previously, 9-cis-retinoic acid (9cRA) was defined as a potent RXR activator. Here we describe Vitamin F (phytanoic acid), a unique RXR effector identified from organic extracts of bovine serum by following RXR-dependent transcriptional activity, and compositions containing Vitamin F.

INTERNATIONAL SEARCH REPORT

Interna . Application No
PCT/US 96/15205

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/00 C12N5/00 A61K31/20 A61K31/23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARCH. SCI. PHYSIOL, vol. 23, no. 4, 1969, pages 437-51, XP000611600 BARAUD, J. ET AL.: "Rôle de la cha*ne phytyle sur certains symptomes d'avitaminose E chez le poussin." see the whole document ---	1-3
Y		6-10
X	C.R.ACAD. SCI. PARIS, SER D, vol. 268, no. 9, 1969, pages 1339-41, XP000611606 BARAUD, J. ET AL.: "Rôle de l'acide phytanique et du phytol chez les poussins en avitaminose e." see the whole document ---	1-3
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 December 1996	11.03.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 96/15205

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. INHER. METAB. DIS., vol. 8, no. suppl 2, 1985, pages 111-112, XP002020040 KRYWAWYCH ET AL.: "The Effect of Phytol upon Skeletal Muscle Damage in Vitamin E-Deficient Animals" see the whole document	6-10
A	HELV. CHIM. ACTA, vol. 41, no. 118, 1958, pages 1038-1052, XP002020041 WEBER: "Ueber den Mechanismus der Reactivierung der Bersteinsäure-cytochrom-c-Reductase durch die Vitamine E und K." see the whole document	1-16
X	JP 58 035 505 A (MASATOSHI TAKENOUCHI) 2 March 1983	1,2,6-8
Y	see abstract	6-10
X	US 5 236 950 A (AOYAMA HAJIME ET AL) 17 August 1993 see the whole document	1,4
X	US 5 221 796 A (MORI HIDETO ET AL) 22 June 1993 see the whole document	1,4,5
A	CAN J. PHYSIOL. PHARM, vol. 50, no. 5, 1972, pages 458-62, XP002020042 HIDIROGLOU: "Fate of 14C-phytol Administered Orally to Sheep" see the whole document	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/15205

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. CLAIMS 1-10 : Use of phytanic or phytenicacid or their ester derivatives for treating a mammal with vitamin F deficiency.
2. CLAIMS 11-15: Composition and method comprising phytanic or phytenic acid and their ester derivatives for culturing mammalian cells.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna. Application No
PCT/US 96/15205

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 58035505 A	02-03-83	NONE	
US 5236950 A	17-08-93	JP 2200614 A	08-08-90
		JP 1308214 A	12-12-89
		AU 2995389 A	24-08-89
		BE 1004398 A	17-11-92
		CH 678810 A	15-11-91
		DE 3904751 A	24-08-89
		FR 2627384 A	25-08-89
		GB 2216003 A,B	04-10-89
		NL 8900396 A	18-09-89
		JP 2076804 A	16-03-90
US 5221796 A	22-06-93	JP 4257544 A	11-09-92
		JP 4257595 A	11-09-92
		JP 4257596 A	11-09-92
		JP 4316586 A	06-11-92